TABLE 3. The lack of influence of age of donors on tolerance induction in B10 mice

Recipient[a]	Group	Spleen cells (5×10^6/g)	CP (200 mg/kg)	B10.BR skin graft survival (days)	No. mice	MST±SD
B10(8w)	1	B10.BR(6w)	−	9, 9, 9, 10, 13	5	10.0±1.7
	2	B10.BR(6w)	+	23, 24, 39, 39, 46, 60, 74, 81, 81, 81, 88	11	57.8±24.5
	3	B10.BR(12w)	−	9, 10, 10, 11, 11	5	10.0±1.0
	4	B10.BR(12w)	+	20, 21, 25, 30, 39, 53, 53, 60, 60, 95	10	45.6±23.4[b]
	5	B10.BR(37w)	−	8, 9, 9, 10, 11	5	9.4±1.1
	6	B10.BR(37w)	+	19, 20, 20, 23, 30, 53, 53, 81, 81, 109	10	48.9±32.1[b]

[a] See footnote[a] of Table 1.
[b] Not significant compared with group 2 by the *U* test.

by complexities related to the tolerogenic spleen cell populations, as already indicated in another study of this series.[3] Namely, in the induction of tolerance with the combined treatment of allogeneic spleen cells plus CP, the antigenicity of the spleen cells, the numbers of T cells, and the numbers of Ia positive cells also may be as crucial as the numbers of the stem cells contained in the preparation.

In summary, the age of adult recipient mice was found crucial to the induction of skin allograft tolerance with allogeneic spleen cells plus CP. By contrast, the age of the donor mice used for tolerance induction did not appear to be crucial for the induction of a tolerant state.

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FK506: A NOVEL IMMUNOSUPPRESSIVE AGENT
CHARACTERISTICS OF BINDING AND UPTAKE BY HUMAN LYMPHOCYTES

Several studies indicate FK506, a novel compound (Fig. 1), to be a potent immunosuppressive agent (*1*). Like cyclosporine, FK506 is isolated from a fungus, *Streptomyces tsukubaensis*, and is hydrophobic. Although cyclosporine is a peptide of larger molecular weight than FK506, both are cyclic compounds, with cyclosporine being more rigid than FK506. Recent in vitro studies suggest that FK506 possesses immunosuppressive properties similar to those of cyclosporine, implying that the mechanism of action for these two drugs may be similar as well. The immunosuppressive effect of FK506 is several-hundred-fold

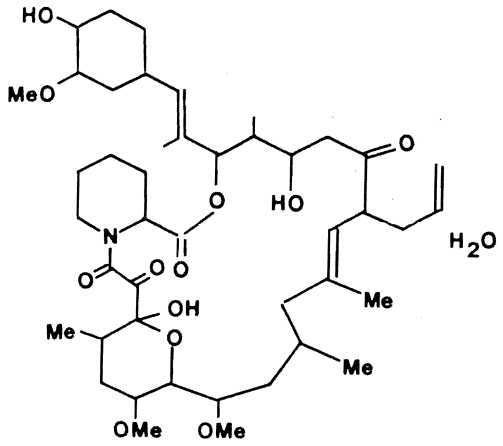

FIGURE 1. FK506.

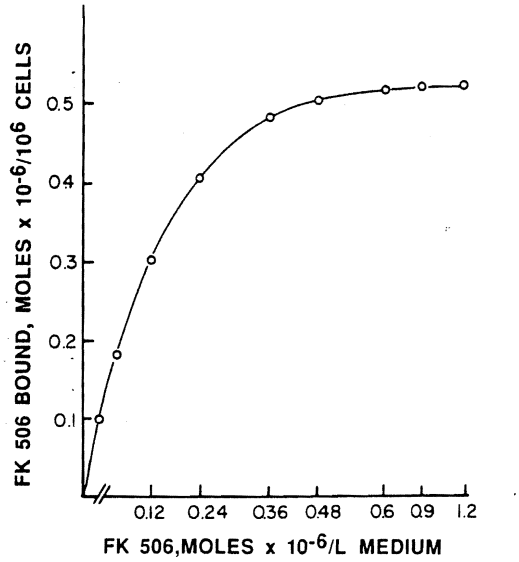

FIGURE 2. Binding of FK506 by human peripheral blood lymphocytes. Normal human lymphocytes (5×10^6) were incubated for 60 min at 37°C in 250 μl of 1% BSA-RPMI 1640 medium containing the indicated concentrations of FK506. The cells were chilled at 0°C for 10 min and washed twice with cold saline, resuspended in phosphate buffer (0.2 M, pH 7.4), and the suspension was used for FK506 determination using the Fujisawa method (*6*). Results are in μmol/10^6 cells.

greater than that of cyclosporine (*2, 3*). As previously demonstrated for cyclosporine, the inhibitory effect of FK506 is seen in mixed leukocyte culture and on secondary proliferation of alloreactive T cells harvested from MLC or propagated from organ transplant biopsies (*4*). The published data further show that immunosuppression by FK506 may be mediated through an inhibition of interleukin-2 release (*2, 4*), again emphasizing the similarities between cyclosporine and FK506.

In this report we describe the characteristics and kinetics of cellular uptake and intracellular binding of FK506 by human peripheral blood lymphocytes. PBL were isolated from the blood of four healthy donors and depleted of their monocyte content as described previously (*5*).

Binding and uptake of FK506 by PBL is shown in Figure 2. The uptake of FK506 by PBL is a saturable process, with saturation occurring at an approximately 0.5 μM concentration of the drug. Scatchard analysis of the binding data is consistent with two distinct classes of binding sites—one with a K_d = $3.9\pm1.8\times10^{-8}$ M for the high affinity sites, and the second with a $K_d = 5.2\pm0.8\times10^{-6}$ M for the low affinity sites. This analysis further reveals that there are $5.6\pm1.0\times10^4$ and $2.5\pm0.9\times10^6$ high- and low-affinity binding sites, respectively, per cell. LeGrue et al. (*7*) have reported that binding of cyclosporine by normal PBL also exhibits two classes of binding sites with a K_d of $2\text{–}6\times10^{-9}$ M for the high-affinity site and a K_d of about 10^{-7} M representing a low-affinity site. They have further indicated that only B cells possess high-affinity sites. Our data, together with this information, seem to further delineate the similarities between FK506 and cyclosporine.

Merker and Handschumacher (*8*) have studied the intracellular localization of cyclosporine by a murine thymoma cell line (BW5147), and have shown that cyclosporine binds to a cytosolic protein (termed *cyclophilin*) with an apparent molecular weight of 15,000–20,000 daltons. More recently, Fabre et al. (*9*) have shown the presence of a similar protein in a human Burkitt lymphoma cell line (RAJI cells). The dissociation constant for the binding of cyclosporine to cyclophilin has been reported to be approximately 2.2 μM. We have investigated the binding of FK506 to cytosolic proteins obtained from PBL. The elution profile of FK506 and cyclosporine on a Bio-Gel P-60 exclusion column that had been calibrated with ovalbumin, carbonic anhydrase, cytochrome C, and vitamin B12 is shown in Figure 3. A graph of the log molecular weight versus the ratio of the elution volume to the void volume for the

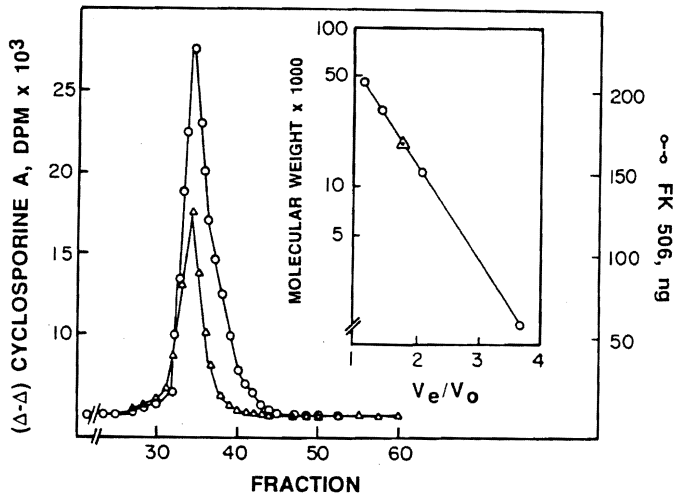

FIGURE 3. Elution profile of the intracellular proteins of PBL on Bio-Gel P-60. Normal human lymphocytes (100×10^6) cells were incubated for 60 min at 37°C in 4 ml 1% BSA-RPMI 1640 medium containing 2 μg FK506 and 1.5 μg (^{3}H) cyclosporine (specific activity 300 dpm/ng). The cells were chilled at 0°C for 10 min, washed twice with cold saline, and resuspended in Tris buffer (20 mM, pH 7.2) containing 2-mercaptoethanol (5 mM) and sodium azide (0.02%). Cells were disrupted by sonic oscillation (*9*), the cellular debris was removed by centrifugation at 40,000 g for 30 min, and the supernate was passed through a Bio-Gel P-6 column to remove protein-free FK506 and cyclosporine. The drugs were then chromatographed on a Bio-Gel P-60 column (40 cm × 1.5 cm) using the same Tris buffer. Utilizing the calibration curve of the molecular weight markers—(a) ovalbumin, (b) carbonic anhydrase, (c) cytochrome C and (d) vitamin B12—as described in the insert, it appears that both cyclosporine (Δ) and FK506 (●) are associated with a protein or proteins with a molecular weight around 18,000–19,000 daltons.

molecular weight markers is shown in the insert. These data suggest that both FK506 and cyclosporine elute in association with a protein with an apparent molecular weight of approximately 18,000–19,000 daltons. Whether this is incidental or there is in fact only one protein that binds both drugs is not clear at present. But, if a single protein, such as cyclophilin, is involved in the binding of both these drugs, then the question of whether two separate binding sites are involved in this process remains to be investigated. Recently, we have demonstrated that human PBL exposed to FK506 for periods varying from 1 hr to 40 hr are able to take up about 20% more cyclosporine relative to control cells not exposed to FK506 (*10*). Similarly, FK506 exposed cells show considerably increased sensitivity to cyclosporine (*2–4*) in terms of mixed lymphocyte reaction and primed lymphocyte tests. This evidence seems to suggest that binding of one drug, for instance FK506, modifies the cellular response to another drug (cyclosporine), resulting in a greater uptake of cyclosporine by the cell.

In summary, kinetics of binding and uptake of FK506 by PBL, and the apparent common association of FK506 and cyclosporine with an intracellular protein, enhances further the similarities between these two immunosuppressive agents.

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AUTOMATED ISOLATION OF MOUSE PANCREATIC ISLETS[1]

The development of an automated method for the isolation of human pancreatic islets (*1*) demonstrated that it is possible to obtain large numbers of intact islets with minimal traumatic action to the islets through a continuous digestion process in which the islets are progressively liberated and saved from the collagenase action. One of the advantages of this procedure is to avoid any chopping, teasing, aspiration through needles, or tissue maceration used in other procedures (*2–8*) that could result in a considerable traumatic action to the islets. In addition, with other isolation procedures, the predigestion of digestion process is generally stopped at an estimated optimal digestion time, and the isolation is then continued by other means. When the pancreatic digestion is interrupted, part of the pancreas is generally still undigested, other portions are overdigested, and what is left is the portion of the preparation that at the time is optimally dissociated: islets free from exocrine tissue but not yet damaged by the collagenase activity.

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The viability of human islets isolated by the automated method has been tested already in different models (*9–11*) that demonstrated the functional integrity of the islets obtained by this procedure. After these encouraging results, it was decided to test a similar procedure for mouse islet isolation in order to determine whether the number of donor mice required to make a recipient normoglycemic could be reduced. Our original collagenase technique (*12*) for mouse islet isolation required 24 mice to obtain approximately 1,200 islets for transplantation. In addition, a small scale model of the automated isolation procedure would be useful for the isolation of islets from other small animals, or for the digestion of fetal or neonatal pancreases of larger mammals, including man.

Six male B10.BR/SgSnJ (B10.BR) mice, 5–8 weeks old, were used for each isolation. Under nembutal anesthesia, the mice were opened by a midline incision. The bile duct was clamped near the duodenum and the pancreas distended by intraductal injection (30-gauge needle) in the proximal common bile duct of 2 ml cold (4°C) Hanks' balanced salt solution containing 1 mg/ml collagenase (Sigma, Type XI). Six pancreases were then